J. Physiol. (1957) 137, 218–244

THE ACTION OF CALCIUM ON THE ELECTRICAL PROPERTIES OF SQUID AXONS

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(*Received* 7 *February* 1957)

Physiologists have been interested in the action of calcium on excitable tissues since the days of Ringer (1883). Some of the main facts established (see Brink, 1954) are that increasing the external calcium concentration raises the threshold, increases membrane resistance (Cole, 1949) and accelerates accommodation. Reducing the calcium concentration has the converse effects, and frequently leads to spontaneous oscillations or repetitive activity (e.g. Adrian & Gelfan, 1933; Arvanitaki, 1939). Other observations which may be less well known are that removal of calcium reduces rectification (*Loligo* nerve, Steinbach, Spiegelman & Kawata, 1944) and increases the fraction of the sodium carrying system which is in a refractory or inactive condition (Purkinje fibres, Weidmann, 1955). In connexion with the last observation, it is interesting that tissues which do not normally give an anode break response can be made to do so by reducing the concentration of calcium ions in the external medium (Frankenhaeuser, 1957). Many of these results may be described by saying that the stabilizing effects of an increase in external calcium are similar to those of an increase in membrane potential, while the excitatory effects which follow its removal resemble those of a reduction in membrane potential (see Brink, Bronk & Larrabee, 1946). This has led to the suggestion that electrical currents may excite by removing calcium ions from sites or carriers in the membrane (see Gordon & Welsh, 1948; Brink, 1954, p. 249; Hodgkin, Huxley & Katz, 1949, p. 389).

Experiments on single fibres, and particularly those on the giant axons of *Loligo*, indicate that the action potential is caused by changes in the permeability of the membrane to sodium and potassium ions and that these are the ions principally concerned with carrying current through the membrane (see Hodgkin & Huxley, 1952*a*). If this is correct, it should be possible to explain

the action of calcium in terms of its effects on the system which controls the sodium and potassium permeability of the membrane. This is the immediate aim of the present inquiry. A more remote objective is to examine the hypothesis that depolarization of the membrane increases sodium permeability by removing calcium ions from specific carriers or sites in the membrane.

The experiments described here were carried out on the giant axons of *L. forbesi* by the voltage clamp method (Hodgkin, Huxley & Katz, 1952). This enables one to measure the current which flows through the membrane when a length of nerve is depolarized and is then held by electronic feed-back with a fixed potential difference across the membrane. In previous work with this technique (Hodgkin & Huxley, 1952*a*, *b*) the concentrations of sodium or potassium were varied, while calcium or magnesium were kept at the concentrations in sea water. In the present experiments the principal variable was the concentration of calcium (or sometimes magnesium) in the external medium, and the concentrations of sodium or potassium were kept at approximately the same value.

METHODS

In most respects the apparatus and method were similar to those described by Hodgkin *et al.* (1952). One difference was that we used a larger cell which allowed axons of greater total length to be employed. The object of this was to obtain axons in better condition. However, as will appear from the next section, the change revealed a weakness in the technique, and in many experiments it was necessary to reduce the total length of the axons in order to avoid the artifacts produced by action potentials set up at the edge of the guard system.

During the course of the work it was found that axons survived better with electrodes which had been used for some time than with ones which had been newly made. It also appeared that coating electrodes with chloride before each experiment did not materially reduce their polarizability inside the axon but did tend to make the electrodes more injurious to the fibres. About half the experiments described here were carried out with a single electrode; this was chlorided when first made, but not before each experiment.

The circuit and performance of the feedback amplifier were as described by Hodgkin *et al.* (1952). Compensated feedback was employed with a setting of $p = 0{\cdot}3$ (cf. p. 341, Hodgkin *et al.* 1952).

Propagation artifacts

One way of testing the voltage clamp method is to set the input of the feedback amplifier at a level which holds the membrane potential at its resting value and then to send a spike into the guard system by stimulating the end of the fibre. Ideally, the feedback system should operate in such a way that no current flows through the central area of membrane and that the potential difference across the latter remains at its resting value. This control was carried out on a few occasions in 1949 with satisfactory results. On repeating the test systematically, it was found that although the feedback amplifier held the recorded membrane potential constant to within less than 0·05 mV, an injected spike often caused a propagation artifact consisting of a brief pulse of inward current of amplitude about 0·1 mA/cm^2. The artifact did not appear unless the ends of the fibre were in good condition, and it is possible that the success of the control experiment in 1949 may have depended on the use of short axons with depolarized ends. The artifact only occurred if the membrane potential was close to the resting level. If the central section was depolarized, the artifact from an injected spike disappeared and did not add to the membrane current. For some time we were unable to see how the artifact arose, since various measurements showed that the current was produced by the feedback amplifier and flowed through the membrane, in spite of the

fact that the membrane potential was kept at its resting value so that no current should flow. This argument would be correct if the axis of the nerve were equipotential over the length occupied by the exposed part of the voltage wire. In practice, the wires are polarizable so that it is possible to have a longitudinal potential gradient along the nerve. If the outer edges of the central region are depolarized and the centre is hyperpolarized the mean potential recorded by the voltage wire will be practically zero, but there can still be a net inward current through the membrane since both hyperpolarization and depolarization may give an inward current. Another way of looking at the phenomenon is to say that because of the polarizability of the current wire, current which flows into it as a result of an injected spike is not confined to the end but tends to be distributed over the whole length of the wire.

Artifacts similar to those described were observed in the present work when the membrane potential was clamped near its resting level and a propagated spike arose at the edge of the guarded section (see, for example, Fig. 14, record 2*a*). This situation occurred rarely in the experiments described previously (Hodgkin *et al.* 1952) but was common in the present work, particularly when using calcium-deficient solutions. Fortunately the artifacts only appeared over a limited range of membrane potentials and could be recognized by their all-or-nothing appearance. The proper cure for the defect might be either to use some less polarizable form of electrode, or to use separate feedback amplifiers and electrodes for the guard and measuring sections. Since there was little prospect of operating either system without much further research, the artifacts were kept small by reducing the length of fibre outside the guard system to about 1 cm or less. This was done by working close to the cannula and by tying off the fibre below the spiral electrode just before the measurements were started. With these precautions, propagation artifacts were reduced to the size shown in Fig. 2, top record, series II. As a further measure, solutions with a reduced sodium concentration were sometimes employed.

Solutions

The solutions used were approximately isotonic with sea water and had the composition given in Table 1. In most cases the sodium concentration was increased as calcium was reduced, but in certain experiments sodium was held constant at a fixed level (for example, 196 or 392 mM); in these solutions isotonicity was preserved with choline chloride. No buffer was added, in order to avoid difficulties with calcium complexes or precipitates. 0·5 mM-HCO_3 was added to all solutions and the final pH was always checked with brom-thymol blue; if necessary the pH was adjusted to about 7·5 with traces of HCl or NaOH.

Preliminary tests showed that axons survived for 12 hr or more in the 112 mM-Ca solution and this was usually employed as the 'normal' or reference solution. Natural sea water at Plymouth contains about 11 mM-Ca and 55 mM-Mg (Webb, 1939). Artificial sea waters made up with these concentrations have been found to have roughly the same stabilizing effect on the squid axon as one containing no Mg and 40–50 mM-Ca.

TABLE 1. Composition of solutions (mg ions/l. solution)

	[Ca]	[Mg]	[Na]	[Choline]	[K]	[Cl]	[HCO_3]	Total
Solutions with [Na] + $\frac{3}{2}$[Ca] = 560 mM	112	0	392	0	10·4	626	0·5	1141
	22	0	527	0	10·4	581	0·5	1141
	4·4	0	553	0	10·4	572	0·5	1141
	0	0	560	0	10·4	570	0·5	1141
Solutions with constant [Na]	112	0	196	196	10·4	626	0·5	1141
	22	0	196	331	10·4	581	0·5	1141
	4·4	0	196	357	10·4	572	0·5	1141
	112	0	392	0	10·4	626	0·5	1141
	22	0	392	135	10·4	581	0·5	1141
	4·4	0	392	161	10·4	572	0·5	1141
Solution with Mg	0	112	392	0	10·4	626	0·5	1141

pH: *ca.* 7·5.

RESULTS

The qualitative effect of $[Ca]_0$ *on inactivation*

Before describing the main experiments it is convenient to consider one result which is discussed in greater detail on p. 232. In Fig. 1, the left-hand column of records shows the membrane currents associated with a depolarization of 40 mV applied to the resting membrane. In the right-hand column, the membrane was hyperpolarized by 40 mV before superposing a cathodal pulse of

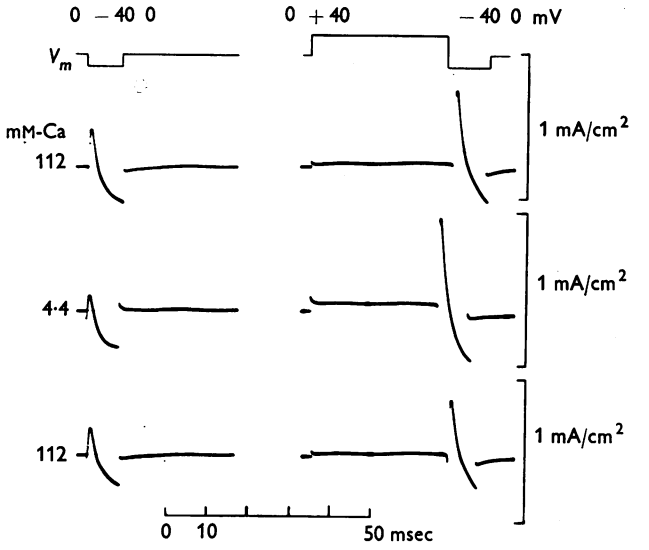

Fig. 1. Membrane currents under a voltage clamp at calcium concentrations of 112 and 4·4 mM. The records show that reducing [Ca] increases the amount of 'inactivation' in an unpolarized fibre. The time course of the membrane potential is given at the top, membrane currents are shown below. Inward current is shown upward in this and all other records. Axon diameter, 680 μ; temperature, 6° C. Solutions with $[Na] + \frac{3}{2}[Ca] = 560$ mM.

80 mV. In both cases the membrane was depolarized to a potential 40 mV below the resting potential during the cathodal pulse. The calcium concentration was first 112 mM, then 4·4 mM and finally 112 mM. About five minutes was allowed for equilibration of the nerve with the external solution, this time having been found more than sufficient for completion of the effects of external calcium on the excitable properties of the membrane.

In all six records the ionic current during the period of depolarization consisted of a transient phase of inward current followed by a phase of outward current. From the evidence of Hodgkin & Huxley (1952*a*, 1953), the former will be regarded as sodium current and the latter as potassium current. With 112 mM-Ca the sodium current without previous hyperpolarization is about

50% of that with polarization. Using the terminology of Hodgkin & Huxley (1952*c*) this means that only about 50% of the sodium-carrying system is readily available and that the remaining 50% is in an inactivated or refractory condition. With 4·4 mM-Ca in the external medium the proportion of the sodium carrying system in an inactive condition is plainly much higher. Thus the inward current in the left-hand column is only about 17% of the inward current in the right-hand column.

Observations similar to those in Fig. 1 were made at the beginning of the investigation and influenced all subsequent experiments. If the effects of calcium on sodium permeability are to be studied, it is plainly most important to start with the nerve in a condition in which the sodium-carrying system has not already been partially converted into a refractory condition. Thus if one compared the records in the left-hand column one might conclude that reducing the calcium concentration decreased the sodium current. On the other hand, it is clear from the right-hand column that reducing the calcium concentration increases the sodium current if the membrane is anodally polarized before applying the cathodal pulse. On p. 232 it is shown that a hyperpolarization of 40 mV is sufficient to remove resting inactivation at all the calcium concentrations (except zero) used in the present work. In most of the experiments the fibre was therefore hyperpolarized by 40–60mV before applying the depolarization.

Another advantage of using anodal polarization was that it stopped the fibre firing repetitively in low calcium solutions. Ideally, switching in the feed-back amplifier should stabilize the membrane, but since the ends of the axon were not under the control of the amplifier, these regions sometimes set up trains of spikes which caused repetitive artifacts in the records of membrane current, in spite of the fact that the recorded potential was held constant to within 0·1 mV. A combination of anodal polarization and feedback stabilized both voltage and current base line.

Quantitative experiments

The effect of $[Ca]_0$ *on the relation between sodium conductance and membrane potential*

Membrane currents. Fig. 2 illustrates the results obtained in the most complete experiment. The records are of the usual kind observed in voltage clamp experiments. Thus the membrane current during a sudden depolarization consists of (1) a brief surge of capacity current of which only the tail can be seen, (2) a phase of sodium current which is inward for cathodal pulses smaller than about 126 mV and outward for larger pulses, (3) a phase of outward potassium current which rises with a marked delay and can be seen uncomplicated by sodium current at the sodium potential (e.g. record 126 mV, series III).

The most obvious result in Fig. 2 is that a reduction of calcium concentration caused a large increase in sodium current for voltage steps of 20–60 mV but that there was less alteration with larger pulses.

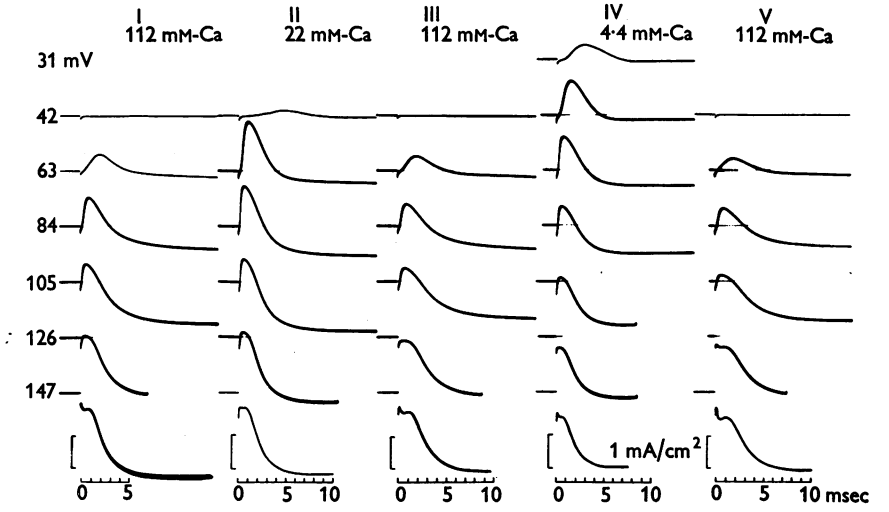

Fig. 2. Membrane currents associated with cathodal steps of 31 to 147 mV superposed on a steady anodal polarization of 41·5 mV. Solutions with $[Na]+\frac{2}{3}[Ca]=560$ mM; axon diameter, 583 μ; temperature, 6° C. Inward current due to the anodal polarization was: series I, $<2{\cdot}5\times10^{-3}$ mA/cm²; series II, 22×10^{-3} mA/cm²; series III, $2{\cdot}5\times10^{-3}$ mA/cm²; series IV, 68×10^{-3} mA/cm²; series V, 17×10^{-3} mA/cm². The following curves have been traced and scaled to the standard amplification from records taken at other amplification: series I, 42 and 63 mV; series II, 42 and 147 mV; series III, 42 and 147 mV; series IV, 31 and 147 mV; series V, 42 and 147 mV; the other curves are photographic reproductions of the original records. The hump of inward current in the top records of series II and series IV is probably a propagation artifact (see p. 219).

$[Ca]_0$ *and the sodium potential*

The first step in analysing records like those in Fig. 2 is to determine the equilibrium potential for the sodium ion, V_{Na}. The procedure adopted was to take V_{Na} as the potential at which the ionic current was flat initially and showed no hump of sodium current (cf. Hodgkin & Huxley, 1952*a*). Since the depolarization was increased in relatively coarse steps, the sodium potential could not be found by direct inspection of records, and it was necessary to interpolate between the two records in which the sodium current changed from inward to outward. The values obtained in this experiment are given in Table 2.

The marked drop in sodium potential between the second and third measurements in 112 mM-Ca (series III and V) suggest that the fibre was gaining sodium rapidly during the intervening period in 4·4 mM-Ca. This is reasonable, because the fibre had a low membrane resistance in this solution and there is evidence in other tissues that low calcium promotes sodium entry (mammalian muscle, Creese & Roberts, 1955; *Sepia* giant axon, Hodgkin & Keynes, unpublished). Since the measurements of V_{Na} were made at the end of the period in each solution the most reliable comparison is between series II and III or between IV and V. The differences in V_{Na} for these tests are -6 and -9 mV. These values are in reasonable agreement with those calculated from the sodium concentrations of the solutions on the assumption that the resting potential remained constant. The

assumption was partially justified in this experiment by the fact that the setting required to keep the feedback amplifier in balance did not have to be reset by more than 1 mV when the solutions were changed.

In experiments with a conventional type of internal electrode (Frankenhaeuser, Hodgkin & Keynes, unpublished), it was found that the resting potential of the squid fibre increased by 3–6 mV as Ca was reduced from 112 to 22 mM; at lower calcium concentrations, if the fibre was not firing spontaneously the resting potential was somewhat variable but was not usually displaced from its normal value by more than 5 mV. The increase in resting potential between 112 and 22 mM-Ca was noticed in some but not all of the voltage clamp experiments, and seems to have been small in the experiment which has just been considered. The discrepancy is tentatively attributed, either to the drastic effects of repeated hyperpolarization or to the difficulty in determining the resting potential with a metal electrode.

TABLE 2. Equilibrium potentials for sodium in the experiment illustrated by Fig. 2

Series	$[Ca]_o$ (mM)	$[Na]_o$ (mM)	V_{Na} (mV)	ΔV_{Na} (observed) (mV)	ΔV_{Na} (calculated from $[Na]_o$) (mV)
(I)	112	392	−97	—	—
(II)	22	526	−101	—	—
(III)	112	392	−95	−6	−7·1
(IV)	4·4	553	−91	—	—
(V)	112	392	−82	−9	−8·3

Potentials are given relative to the resting potential, depolarization negative.

$[Ca]_o$ *and the relation between sodium conductance and membrane potential*

The sodium conductance (cf. Hodgkin & Huxley, 1952*a*) is defined by the relation

$$g_{Na} = \frac{I_{Na}}{V - V_{Na}},$$

where g_{Na} is the sodium conductance per cm² of membrane,

I_{Na} is the sodium current density,

V is the membrane potential,

V_{Na} is the equilibrium potential for the sodium ions.

The peak sodium conductance at any depolarization can be estimated without much uncertainty from records such as those in Fig. 2. The membrane potential, V, was controlled by the feedback amplifier and was determined by direct measurements at the end of the experiment. V_{Na} was obtained in the manner described in the previous section. The peak sodium current was measured as shown inset in Fig. 3.

The results of this analysis are given in Fig. 3, which shows the peak sodium conductance on a logarithmic scale plotted against the displacement of the membrane potential from its resting value. The main effect of reducing the calcium concentration is to shift the $g_{Na} - V$ curve along the voltage axis in a direction such that a smaller depolarization is required to produce a given rise in sodium conductance. The shifts obtained in three experiments of this type are given in Table 3 under the heading ΔV_c. The figures in Table 3 were

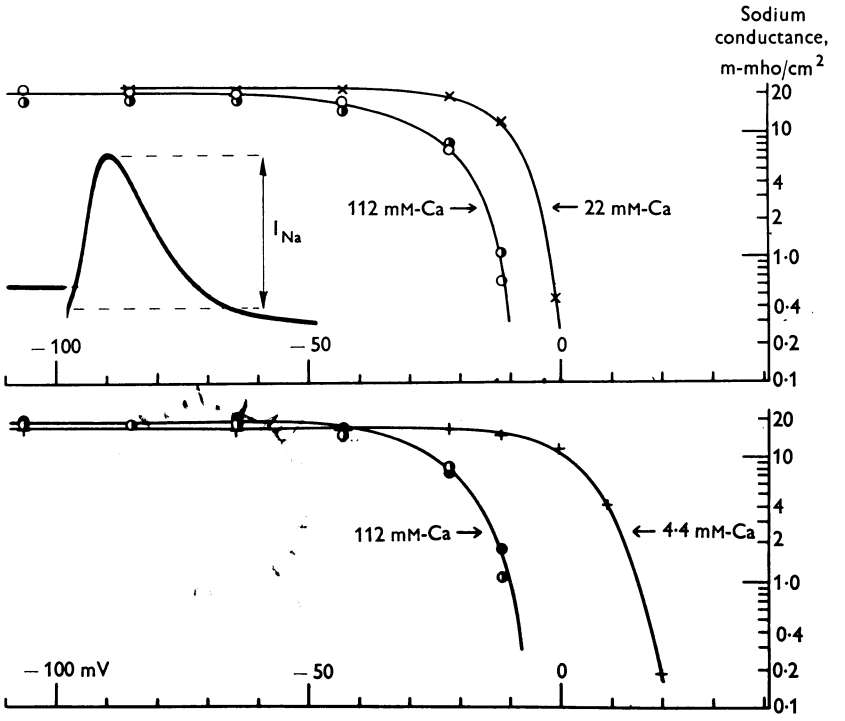

Fig. 3. Peak sodium conductance plotted against the displacement of the membrane potential from its resting value. From the same experiment as Fig. 2. ○, series I; ×, series II; ◑, series III; +, series IV; ●, series V. The peak sodium conductances were measured shortly after a sudden depolarization from a steady hyperpolarization of 41·5 mV. The method of measuring I_{Na} is shown inset.

TABLE 3. Effect of $[Ca]_0$ on relation between sodium conductances and membrane potential in five experiments

V_c, potential at which $g_{Na} = \frac{1}{10}$ maximum (mV)

$[Ca]_0$ (mM)	(1) V_c	(1) ΔV_c	(2) V_c	(2) ΔV_c	(3) V_c	(3) ΔV_c	(4) V_c	(4) ΔV_c	(5) V_c	(5) ΔV_c	Mean ΔV_c
112	−17	—	−25	—	−15	—	−24·5	—	—	—	—
22	− 3·5	13·5	− 3	19	− 4	10·5	− 6	17·5	—	—	15
112	−17	—	−19·5	—	−14	—	−22·5	—	−24	—	—
4·4	+20·4	36	+15	33	+13	26	—	—	+ 1	25	30
112	−15	—	−16	—	−12	—	—	—	−24	—	—

Expt.	Solutions with	Hyperpolarization before cathodal pulses (mV)
(1)	Na + $\frac{3}{2}$ Ca = 560	+56
(2)	Na + $\frac{3}{2}$ Ca = 560	+56
(3)	Na + $\frac{3}{2}$ Ca = 560	+41·5
(4)	Na = 196	+40
(5)	Na = 196	+39

Potentials are given relative to the resting potential (depolarization negative).

calculated on the assumption that the resting potential was the same in all three solutions; since there was some doubt about this, the values for ΔV_c were recalculated using the sodium potentials as a base line (allowance was made for differences in external sodium concentration). The mean values for ΔV_c obtained by this method were in good agreement with those in Table 3, the figures being 15 mV between 112 and 22 mM-Ca or 29 mV between 112 and 4·4 mM-Ca.

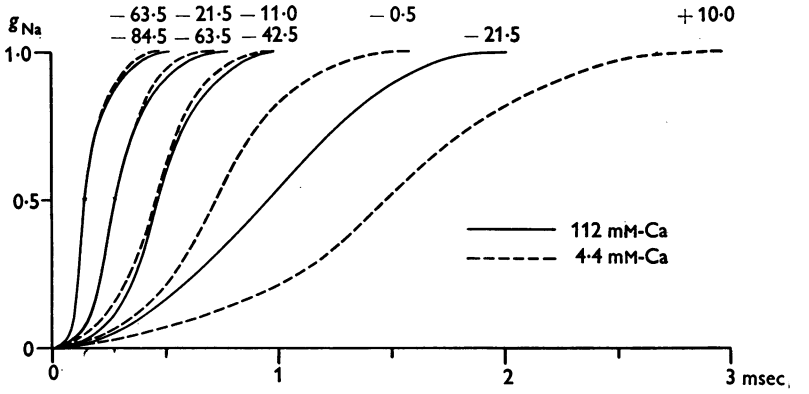

Fig. 4. Increase of sodium conductance plotted against time after onset of cathodal polarization. The figures at the ends of the curves indicate the displacement of the membrane potential from its resting value. The sudden displacements were made from an anodal polarization of 41·5 mV. The upper row corresponds to the curves in 4·4 mM-Ca, the lower to the curves in 112 mM-Ca. From the same experiment as Fig. 2.

$[Ca]_0$ and the rate of rise of sodium conductance

From the records in Fig. 2 it is evident that reducing the calcium concentration not only increases the maximum sodium current, but also raises the rate at which the sodium current increases at the beginning of the period of depolarization.

Fig. 4 gives the family of curves obtained by calculating sodium conductance as a function of time; these curves have been scaled to the same amplitude since the absolute conductance was difficult to determine when V was close to V_{Na}. It is evident that the rate of rise of sodium conductance is greatly increased by removing calcium and that the maximum conductance is reached earlier in the low calcium solution.

The superposed curves in Fig. 4 show that the $g_{Na}-t$ curve in 112 mM-Ca at a given depolarization is roughly equivalent to the curve in 4·4 mM-Ca at 20–40 mV less depolarization. Similar agreement was obtained with 22 mM-Ca, the shift in this case being 10–20 mV. As with the peak sodium conductance, it appears that depolarization and removal of Ca are roughly equivalent. In both cases the shift is of the order of 15 mV for a fivefold reduction of Ca.

$[Ca]_0$ and the rate at which sodium conductance shuts off under an anode

Hodgkin & Huxley (1952*b*) showed that the sodium conductance decreases rapidly if the membrane is repolarized at any stage during the period of raised sodium conductance. This 'shutting-off' is quite distinct from the relatively slow inactivation which occurs if the depolarization is maintained. In the former case the sodium-carrying system is returned to the resting state and is capable of transporting sodium at a high rate if the nerve is again depolarized. In the latter case a second pulse of depolarization is ineffective until the state of inactivation or refractoriness has worn off.

If calcium ions stabilize the membrane by occupying sites or combining with carriers, one would expect that the rate at which the sodium conductance shuts off might be accelerated by increasing the calcium concentration. The first results to be described are those in which a brief cathodal pulse of amplitude about 170 mV was superposed on a steady anodal polarization of about 40 mV. The duration of the pulse was such that the membrane was repolarized near the moment of maximum sodium conductance. Under these conditions the sodium conductance rises along an S-shaped curve during the period of depolarization and declines rapidly along an exponential curve when the fibre is repolarized. The currents resulting from these changes are shown in Fig. 5. During the cathodal pulse the sodium current is outward because the membrane is depolarized beyond the sodium potential by 38 mV. When the membrane is repolarized, the membrane potential is 130 mV above the sodium potential so that there is at first a very large inward sodium current. In 112 mM-Ca the inward current is of short duration because the sodium conductance shuts off rapidly. In 22 mM-Ca the rate is clearly much slower and the exponential tail of current has a longer duration. In 4·4 mM-Ca there was a further prolongation and the tail of inward current then ceased to be exponential.

The effects seen in these records are shown in a simpler manner by calculating sodium conductance from the currents. These curves which are given in Fig. 6 show that the rate at which the sodium conductance is shut off under an anode is greatly reduced by lowering the calcium concentration in the external medium.

Table 4 gives the quantitative results obtained in seven experiments of this type. The conclusion is that reducing the calcium concentration from 112 to 22 mM slows the 'shutting-off' rate by a factor of nearly 5. A reduction to 4·4 mM slows the rate by a factor of 10–15. In this case, the 'shutting-off' rate becomes comparable with the rate at which the sodium conductance is inactivated when the depolarization is maintained, and it is conceivable that an even greater effect might be obtained if this could be allowed for.

It will be seen from Table 4 that the effect of reducing the calcium concentration on the shutting-off rate is much greater than that expected on the simple

principle that a fivefold reduction is equivalent to a depolarization of 10–20 mV (pp. 240–241). In Expt. 3, Table 4, the time constant in 112 mM-Ca with the membrane hyperpolarized 41 mV was about 90 μsec, whereas it was about 260 μsec in 22 mM-Ca with the membrane hyperpolarized 62 mV.

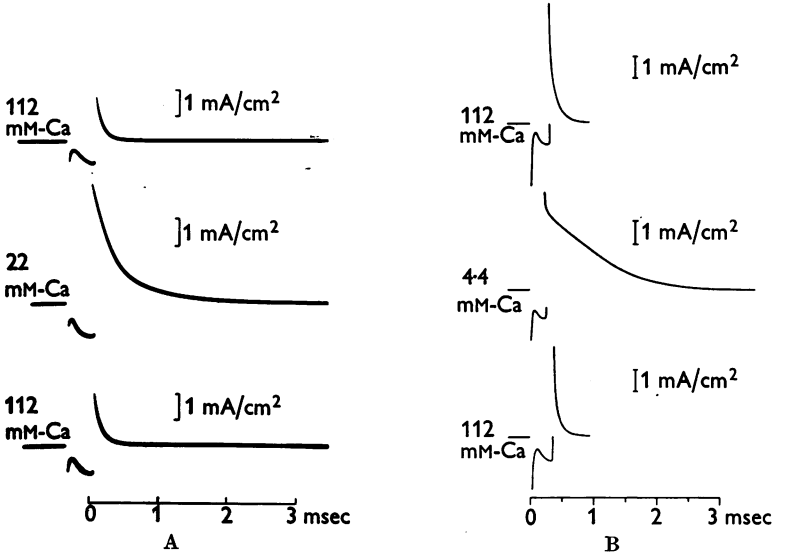

Fig. 5. (A) Records of membrane current associated with a rectangular cathodal pulse of amplitude 168 mV, superposed on a steady anodal polarization of 41 mV. The cathodal pulse ended at the peak of the outward sodium current. $[Ca]_0$ as marked. Solutions with [Na] + $\frac{3}{2}$ [Ca] = 560 mM; axon diameter, 648 μ; temperature, 5° C. (B) Similar to *A* but using 4·4 mM-Ca as the test solution. Axon diameter, 624 μ; temperature, 6·5° C. The records have been traced and scaled to a common time axis. The two axons used in *A* and *B* were from the same squid.

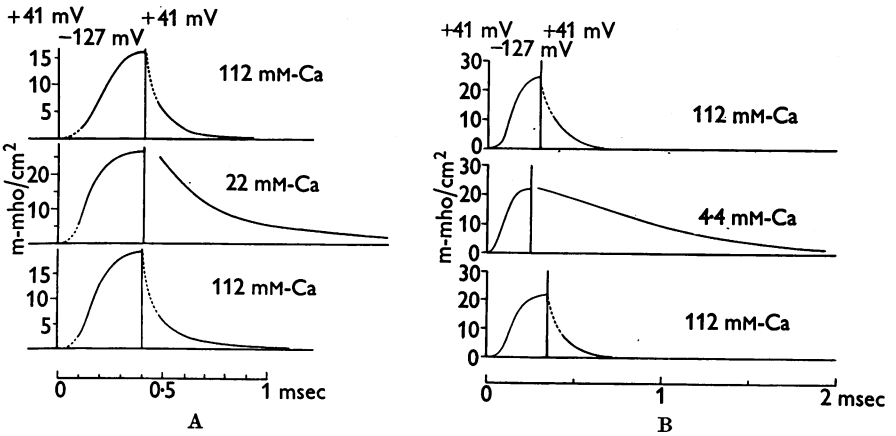

Fig. 6. Changes in sodium conductance plotted against time, calculated from the corresponding records in Fig. 5.

TABLE 4. Effect of $[Ca]_0$ on the time constant with which the sodium conductance decreases under an anode

	112 mM-Ca					22 mM-Ca					4·4 mM-Ca					
Expt.	V (mV)	Pulse (mV)	Temp. (° C)	τ_1 (μsec)	τ_2 (μsec)	V (mV)	Pulse (mV)	Temp. (° C)	τ_1 (μsec)	τ_2 (μsec)	V (mV)	Pulse (mV)	Temp. (° C)	τ_1 (μsec)	τ_{2a} (μsec)	τ_{2b} (μsec)
Experiments with $[Na]+\frac{3}{2}[Ca]=560$ mM																
(1)	+56	−112	4·2	67	95	+56	−112	4·2	600	480	—	—	—	—	—	—
	+56	−112	4·5	77	110	—	—	—	—	—	+56	−112	4·8	1010	1180	1030
	+56	−112	5·2	62	90	—	—	—	—	—	—	—	—	—	—	—
(2)	+47	−123	5·3	80	99	+47	−123	5·2	465	370	—	—	—	—	—	—
	—	—	5·4	82	78	—	—	—	—	—	—	—	—	—	—	—
(3)	+62	−126	5·2	73	59	+62	−126	5·8	245	281	—	—	—	—	—	—
	+62	−126	5·9	104	52	—	—	—	—	—	—	—	—	—	—	—
	+41	−127	4·9	101	73	+41	−127	5·4	353	454	—	—	—	—	—	—
	+41	−127	5·8	112	81	—	—	—	—	—	—	—	—	—	—	—
(4)	+62	−126	6·2	58	67	—	—	—	—	—	+62	−126	6·2	1200	1470	640
	+62	−126	6·9	57	72	—	—	—	—	—	—	—	—	—	—	—
	+41	−127	6·2	104	103	—	—	—	—	—	+41	−127	6·2	1030	1360	570
	+41	−127	6·9	77	108	—	—	—	—	—	—	—	—	—	—	—
Mean	+51·9	−122·6	5·6	81	83·5	+51·5	−121·5	5·0	416	396	+53	−122	5·7	1080	1340	750
Experiments with constant [Na]																
[Na] = 392 mM																
(5)	+52	−123	7·0	—	91	+52	−123	7·3	—	314	—	—	—	—	—	—
	—	—	7·8	—	112	—	—	—	—	—	—	—	—	—	—	—
(6)	+40	−66	7·8	—	159	—	—	—	—	—	+40	−66	7·2	—	650	530
	—	—	9·2	—	173	—	—	—	—	—	—	—	—	—	—	—
[Na] = 196 mM																
(7)	+40	−107	8·8	—	88	+40	−107	7·1	—	196	—	—	—	—	—	—
	+40	−107	7·2	—	72	—	—	—	—	—	—	—	—	—	—	—
	+40	−44	9·3	—	93	+40	−44	7·4	—	257	—	—	—	—	—	—
	+40	−44	6·8	—	68	—	—	—	—	—	—	—	—	—	—	—

Membrane potentials given relative to resting potential, depolarization negative. τ_1 is the line required for the conductance to fall to 1/e of the value immediately before the fibre was repolarized. τ_2 is the value obtained by plotting the tail of current on semilogarithmic paper and fitting a straight line to the points. In 4·4 mM-Ca, where the decline in sodium conductance was not exponential, the initial and final slopes on a semilogarithmic plot are given by τ_{2a} and τ_{2b} respectively.

Variation of 'shutting-off' rate in low calcium

The relatively simple results illustrated in Table 4 are complicated by an effect which makes it difficult to describe the action of calcium in quantitative terms.

When the fibre was in 112 mM-Ca or in sea water containing 11 mM-Ca and 55 mM-Mg, the rate constant of the shutting-off process did not vary much if the fibre was repolarized at different times. This is illustrated by Fig. 7A and C, which show the exponential tails of sodium current in 112 mM-Ca. These curves do not have exactly the same time constant, but the range of variation is not large, as may be seen from Table 5 *A* and *C*. Fig. 7B shows a similar set of curves with 22 mM-Ca in the external solution. From these and from Table 5 *B* it is clear that the time constant varies

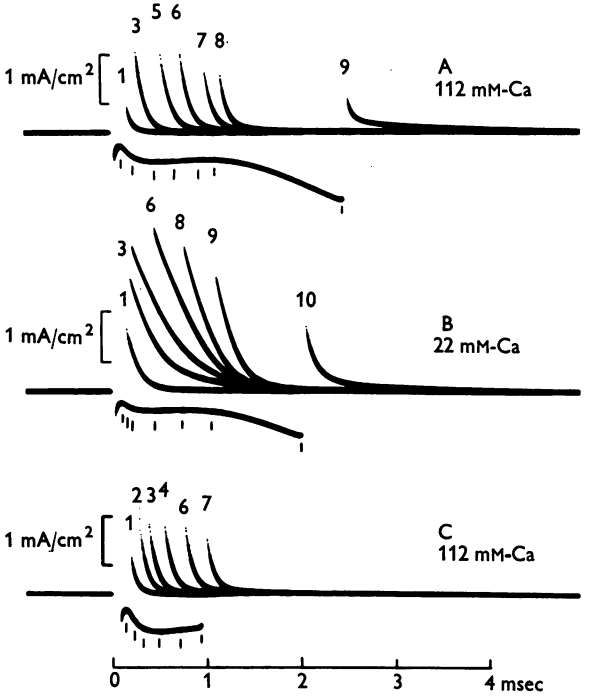

Fig. 7. Records of membrane currents associated with short rectangular cathodal pulses superposed on a steady anodal polarization of +41 mV. The amplitude of the cathodal pulses was 170 mV. Cathodal pulses ended at the times indicated by short vertical lines. This figure is made by photographic superposition of several of the original records. Numbering of the curves is the same as for Table 5. Axon diameter, 805 μ, temperature, 5·3° C.; solutions with $[Na]_0 + \frac{2}{3}[Ca]_0 = 560$ mM. Note that the early part of each curve is obscured by capacity current and that the maximum currents seen here are not proportional to the extrapolated initial conductances given in Table 5.

from 100 to 400 μsec and is greatest a little before the moment of maximum conductance. There appear to be two separate effects, namely that the time constant is relatively short if the sodium conductance has not been fully turned on and that it is also shorter when the conductance is reduced by inactivation. The reason for supposing there are two effects is that inactivation seems to shorten the time constant more than incomplete turning on. Thus in Table 5 *B* the time constant in curve 9 is shorter than in curve 3, although both start at about the same initial conductance.

Because of these complications it is necessary to qualify the conclusions drawn in the preceding section by saying that effects as large as those in Table 4 are only seen if the sodium conductance is fully turned on at the moment when the fibre is repolarized.

A similar conclusion was reached in an experiment in which the membrane was depolarized only to a small extent. In this experiment the axon was not hyperpolarized and depolarizations of about 25 mV were employed. The time constants at 4° C were about 130 μsec in 112 mM-Ca, 200 μsec in 22 mM-Ca and 470 μsec in 4·4 mM-Ca. From the setting of the feed-back amplifier there was evidence that the resting potential rose by 5 mV in the 22 mM-Ca solution, which may account for the small change in time constant with this solution. Although the time constant with 4·4 mM-Ca was

TABLE 5. Time constants in experiment of Fig. 7

Curve	Duration of depolarization (μsec)	Initial conductance (extrapolated) (m-mho/cm^2)	Time constant (μsec)
	A. 112 mM-Ca		
2	229	11·5	86
3	257	15·4	86
4	382	20·6	99
5	476	17·7	99
6	666	15·9	102
8	925	11·7	96
	B. 22 mM-Ca		
1	124	12·8	109
2	152	13·9	185
3	172	17·8	310
4	229	18·7	359
5	238	20·3	401
6	476	22·4	330
7	554	21·2	281
8	764	21·2	256
9	1080	19·4	193
10	2020	8·5	179
	C. 112 mM-Ca		
2	240	11·8	76
3	324	22·0	74
4	420	21·0	84
5	496	24·9	78
6	726	16·8	105
7	915	15·7	86

The initial conductances were obtained by extrapolating the curves reproduced in Fig. 7 to the time at which the fibre was repolarized.

3–4 times longer than in 112 mM-Ca the effect is relatively small compared to most of those in Table 4. A possible explanation of the difference is that if the sodium conductance is not fully turned on, calcium ions may not move far from the sites which they normally occupy. In this case the shutting-off process might occur by calcium ions returning to sites rather than by fresh calcium ions going on from the external solution. The shortening of the shutting-off time constant as inactivation proceeds is a puzzling effect which might have something to do with the fact that there may be less 'sodium carrier', or that the carrier may be changed chemically under these conditions.

Effect of $[Ca]_0$ *on inactivation in the steady state*

Previous experiments have shown that the fraction of the sodium-carrying system which is in a refractory or inactive condition varies with membrane

potential in a characteristic manner (Hodgkin & Huxley, 1952*c*; Weidmann, 1955). In the steady state, the fraction which is not refractory is related to membrane potential by an S-shaped curve of the type shown in Fig. 8. The ordinate in this graph is proportional to the sodium current produced by a test pulse in which the membrane is depolarized to a fixed value, the abscissa is the membrane potential before applying the test pulse. Under an anode the sodium-carrying system is almost wholly in a resting state and is capable of transporting sodium at a high rate when the fibre is depolarized. Under a cathode the

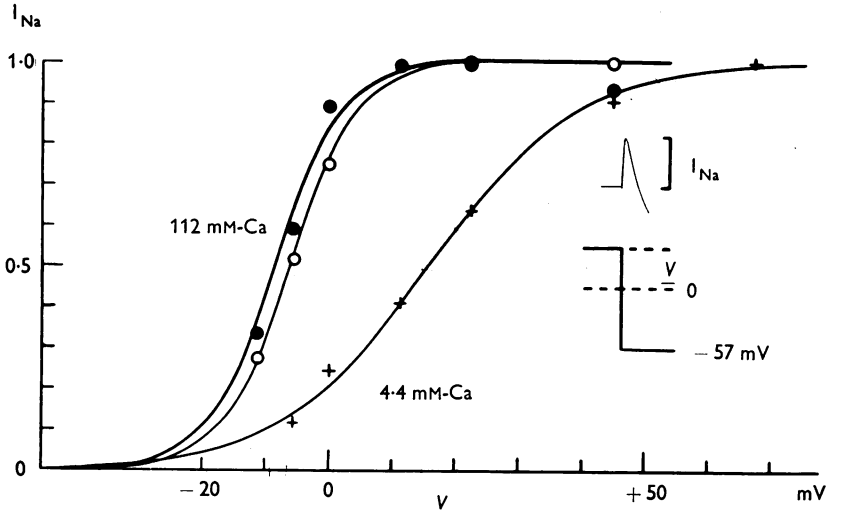

Fig. 8. Inactivation curve in 112 and 4·4 mM-Ca solutions. Abscissa: membrane potential (V) before the cathodal polarization is applied, measured from the resting potential. Ordinate: sodium current associated with a depolarization of 57 mV below resting potential. I_{Na} is scaled in units of maximum current in the two solutions. ○ in 112 mM-Ca; + in 4·4 mM-Ca, ● again in 112 mM-Ca. Fibre diameter, 630μ; temperature, 6·5° C. The curves were drawn from equation 1 of Hodgkin & Huxley (1952*c*) with the values given under (3) in Table 6. The method of measuring I_{Na} is shown inset and, in more detail, by the inset on Fig. 3.

membrane is largely in the refractory state and does not give an increase in sodium permeability when depolarized. As found by Weidmann (1955) in the Purkinje fibres of the mammalian heart, the inactivation curve is shifted along the voltage axis by changing the external calcium concentration; the direction of the effect is that reducing the calcium concentration decreases the fraction of the sodium-carrying system which is readily available and increases the fraction in a refractory or inactive condition. The shift in the inactivation curve is illustrated by the difference between the crosses and circles in Fig. 8. In this experiment a 25-fold reduction of calcium concentration flattened the curve, as well as shifting it laterally by about 20 mV. Similar effects were observed in two other experiments, but the flattening was less marked than in

Fig. 8. Values for the constant k which determines the steepness of the curve are given in Table 6 and may be seen to be similar to that obtained by Hodgkin & Huxley (1952*c*), i.e. 7 mV with 11 mM-Ca, 55 mM-Mg.

Table 6 shows the effect of $[Ca]_0$ on the potential at which inactivation is 50% complete and on the steepness of the inactivation curve. A comparison with Table 3 suggests that calcium ions may have a smaller effect on the inactivation curve than on the relation between sodium conductance and membrane potential. Further experiments are needed to establish this conclusion and to replace the preliminary results in Table 6 by more accurate data.

TABLE 6. Effect of $[Ca]_0$ on relation between inactivation and membrane potential in steady state in three experiments

$[Ca]_0$	V_h (mV)			ΔV_h mean	k (mV)			k mean
(mM)	(1)	(2)	(3)	(mV)	(1)	(2)	(3)	(mV)
112	−2	−1	−5	—	6	5·6	5·2	5·6
22	+9	+1*	−3*	+5	10	5·4	5·2	6·9
112	−2	−0·5	−6	—	5	5·8	5·2	5·3
4·4	+18	+7·5*	+14	+16	8	7·4	11·7	9·0
112	−2	−0·5	−8	—	5	5·8	5·2	5·3

V_h and k were obtained by fitting experimental points such as those in Fig. 8 with the equation used by Hodgkin & Huxley (1952*c*),

$$(h) = 1/(1 + \exp(V_h - V)/k),$$

V_h is the membrane potential at which there is 50% inactivation. V_h is given relative to the resting potential in each solution (depolarization negative). In the cases marked with an asterisk there was evidence from the setting of the feedback amplifier that the resting potential increased by about 5 mV in the low calcium solutions. Temperature about 6° C; solutions with $[Na]_0 + \frac{2}{3}[Ca]_0 = 560$ mM.

Effect of $[Ca]_0$ *on the kinetics of the inactivation process*

This problem was not studied systematically. Such experiments as we have indicate:
(1) that reducing $[Ca]_0$ may increase the rate of inactivation under a cathode;.
(2) that reducing $[Ca]_0$ decreases the rate at which inactivation is removed under an anode.
Both effects would tend to shift the inactivation curve in the manner shown in Fig. 8.

The evidence for the first statement is that, at the same membrane potential, the duration of the phase of inward current is shorter in low calcium than in high (Fig. 2). With relatively weak depolarization this cannot be attributed to a rise in potassium permeability because the outward current in records such as those with 42 and 63 mV pulses in Fig. 2 is small compared to the inward current. With large depolarizations there is no clear evidence about the effect of $[Ca]_0$ on inactivation rate, and it is possible that the limiting rate reached at large depolarizations may be unaffected by calcium. This is what one would expect if the effect of calcium removal were similar to a depolarization.

Table 7 gives the effect of a 25-fold reduction of calcium concentration on the time constant with which inactivation is removed under an anode. It will be seen that increasing the calcium concentration from 4·4 to 112 mM increases the rate of removal of inactivation by a factor of about 2·3. The table also shows that the rate of removal in 4·4 mM-Ca with a hyperpolarization of 56 mV is about the same as the rate in 112 mM with a hyperpolarization of 32 mV.

TABLE 7. Effect of $[Ca]_0$ on rate of removal of inactivation under an anode

Anodal polarization (mV)	$[Ca]_0$ (mM)	τ_h (msec)
56	112	1·05
56	4·4	2·42
56	112	1·18
32	112	2·42

Same axon as Expt. 2 of Table 6: temperature 6° C.

Effect of $[Ca]_0$ *on relation between potassium conductance and membrane potential*

A striking property of cephalopod or crustacean axons is that the outwardly directed current associated with a steady depolarization of 10–50 mV is very much greater than the inward current associated with a corresponding hyperpolarization (Cole & Curtis, 1941; Hodgkin & Rushton, 1946). This

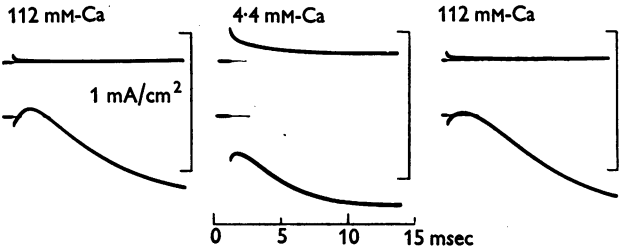

Fig. 9. Membrane currents associated with sudden changes in membrane potential of +42 mV (anodal polarization, upper curve) or −42 mV (cathodal polarization, lower curve). These pulses were applied to a resting fibre in presence of 112 or 4·4 mM-Ca. Solutions contained 196 mM-Na and choline; fibre diameter, 630 μ; temperature, 6·2° C.

rectification has been shown to arise from changes in the potassium conductance of the membrane (Hodgkin & Huxley, 1952*b*, 1953). It has also been shown that the potassium conductance does not increase instantaneously but with a delay of the order of a millisecond along a markedly S-shaped curve. Steinbach *et al.* (1944) reported that the rectification depends on the presence of calcium ions in the external medium; this was confirmed in unpublished experiments carried out by one of us with Professor Katz in 1948. The problem was not studied systematically in the present investigation, but a number of observations indicate that calcium changes the relation between potassium conductance and membrane potential in much the same way as it alters the relation between sodium conductance and membrane potential.

The qualitative effect of reducing calcium is illustrated by Fig. 9. This shows the membrane current produced when steps of ± 42 mV were applied to a resting fibre with either 112 or 4·4 mM-Ca in the external medium. In

contrast to most other records in this paper, the membrane was not hyperpolarized before applying the pulse. In this experiment the external medium contained 196 mM-Na so that the sodium currents are relatively small, particularly in low Ca where inactivation is increased. With 112 mM-Ca in the external medium an anodal step of 42 mV gave a current which was barely visible at the gain employed. The initial ionic current associated with the cathodal step was also small but rose with a delay towards a steady level of about 0·6 mA/cm^2 (the final part of the rise is not shown in these records which were taken for another purpose).

With 4·4 mM-Ca both anodal and cathodal currents were altered in a manner which suggests that the resting potassium conductance was closer to its maximum value. Thus the initial step of ionic current was larger in low calcium and under a cathode the conductance rose with less delay to its maximum value. With anodal pulses, the current in 4·4 mM-Ca was large initially but fell to a low value during the pulse. A similar effect was probably present in high calcium, but even at high amplification the low value of the resting potassium conductance made it difficult to see much decline in current.

Analysis of other records from the experiment of Fig. 9 indicated that with 4·4 mM-Ca the potassium conductance at the resting potential was about 25% of its maximum value. With 112 mM-Ca in the external medium, a depolarization of about 30 mV was required to increase the potassium conductance to a corresponding level.

We have no data from which to estimate the potassium conductance in the manner described previously (Hodgkin & Huxley, 1952*a*, *b*). A rough idea of the effect of calcium on the relation between g_K and V is obtained by plotting I'/V against V, where I' is the 'steady state' current measured after about 10 msec. I'/V approximates to g_K when the latter is large but the method is inaccurate near the resting potential, since other ions besides potassium contribute substantially to the current. However, the general form of the curves in Fig. 10 confirms the impression that a fivefold reduction of calcium shifts the $g_K - V$ curve about 10–15 mV along the voltage axis.

Since the potassium current can be seen uncomplicated by sodium current at the sodium equilibrium potential it was not difficult to investigate the effect of calcium on the time course of the potassium conductance at this point. Fig. 11 indicates that raising the calcium concentration decreases the rate of rise of potassium conductance and delays the onset of the rise. Both effects are qualitatively similar to those produced by increasing the membrane potential, i.e. making the inside of the fibre more negative. Thus the curves of Hodgkin & Huxley (1952*d*) show that the potassium conductance increases more rapidly as the depolarization is increased. In order to explain the marked effect of Ca in delaying the onset of the rise in potassium conductance it is necessary to

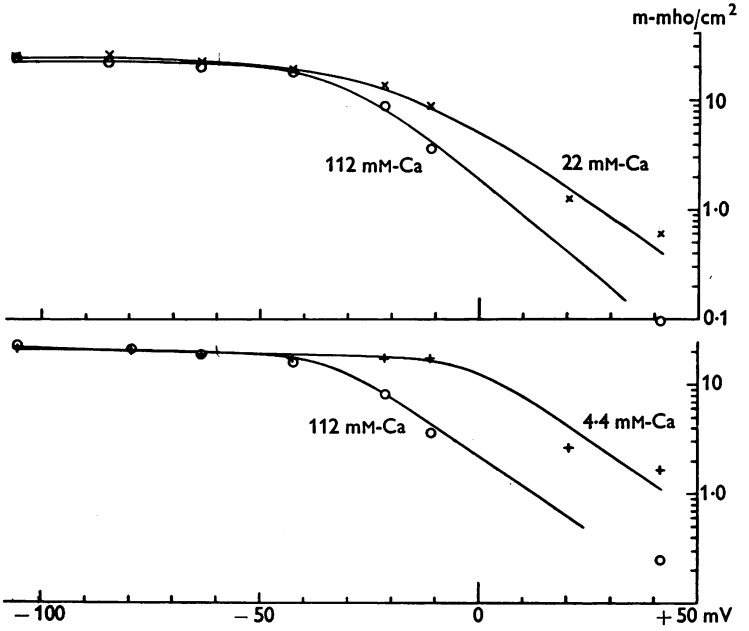

Fig. 10. Effect of calcium concentration on the relation between potassium conductance and membrane potential. Abscissa, displacement of membrane potential from its resting value; ordinate, 'steady state' conductance measured about 10 msec after the beginning of cathodal pulses applied to a fibre hyperpolarized by 41·5 mV. From the same experiment as Fig. 2. Axon diameter, 583μ; temperature, 6° C. Note that the 'steady state' conductance is not exactly equal to the potassium conductance and that, owing to the existence of a leak conductance (Hodgkin & Huxley, 1952*c*), the curves shown here are flatter than the true $g_K - V$ curves.

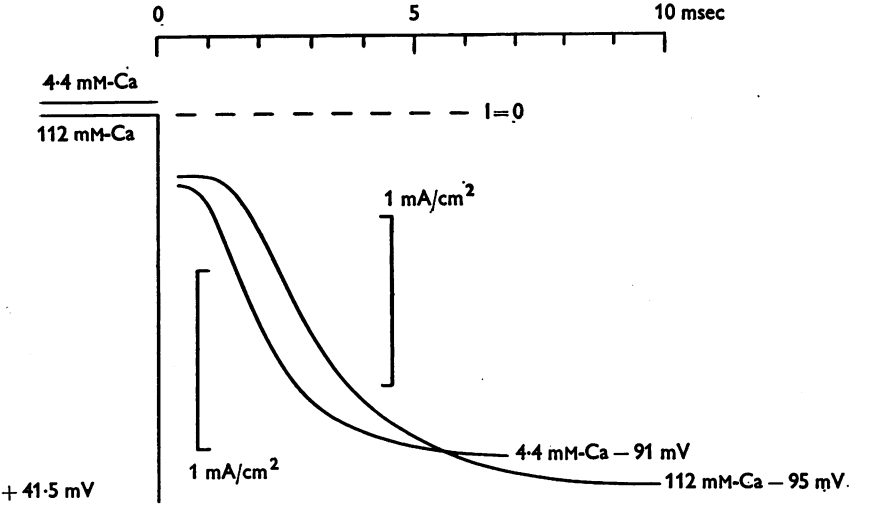

Fig. 11. Membrane currents when the membrane potential is changed from a value 41·5 mV above the resting potential to the sodium equilibrium potential, −95 mV in 112 mM-Ca and −91 mV in 4·4 mM-Ca. The curves are interpolated from a number of records taken at depolarizations close to V_{Na}. From the same experiment as Fig. 2. Axon diameter, 583μ; temperature, 6° C.

consider the state of the fibre *before* the pulse is applied. From this point of view the best comparison to make is between fibres depolarized to the sodium potential from different initial levels. This is illustrated by Fig. 12, which shows that the effect of increasing the membrane potential before the pulse resembles that of increasing calcium concentration, in that both increase the delay in the rise of potassium conductance.

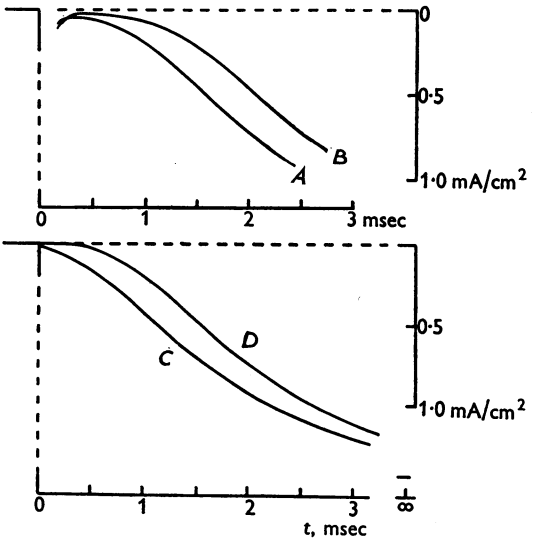

Fig. 12. Effect of previous hyperpolarization on the rise of potassium current. The upper curves show the membrane current when the fibre is depolarized to the sodium equilibrium potential (− 100 mV) from the resting potential (*A*) or from 40 mV anodal polarization (*B*); 112 mM-Ca in both cases. Axon diameter, 678 μ; temperature, 8° C. The lower curves give the potassium conductance calculated by the equations of Hodgkin & Huxley (1952*d*) for conditions similar to those in the upper part of the figure. Curve *C* is for a depolarization from $V = 0$ to $V = -100$ mV; curve *D* for a depolarization from $V = +40$ mV to -100 mV. These curves are appropriate to a temperature of 6° C and a nerve in sea water (11 mM-Ca, 55 mM-Mg). Standard values of V_K, α_n and β_n were used but the value of $\bar{g}_K$ was taken as 15 m-mho/cm² instead of 36 m-mho/cm². The equations used in plotting were $I_K = -1{\cdot}68\ (0{\cdot}9617 - 0{\cdot}6440 \exp -t/1{\cdot}069)^4$ for curve *C* and $I_K = -1{\cdot}68\ (0{\cdot}9617 - 0{\cdot}9456 \exp -t/1{\cdot}069)^4$ for *D*. The experimental curves were obtained on a fibre which had previously been exposed to a solution containing 4·4 mM-Ca; this may account for the relatively small current in *A* and *B*.

The effect of anodal polarization in delaying the rise of potassium conductance (which has not been observed previously) fits in well with the formulation of Hodgkin & Huxley (1952*d*). This is illustrated by recalculating the rise in potassium conductance with g_K (or n) initially very small instead of at the resting level. The curve for $n = 0{\cdot}0161$, which is appropriate to an initial hyperpolarization of 40 mV, is shown in Fig. 12*D* and may be seen to rise with a much longer delay than the corresponding curve for no initial polarization (curve *C*, $n = 0{\cdot}3177$).

Calcium-free solutions

Calcium-free solutions are difficult to work with because (1) the fibre is liable to be damaged irreversibly by prolonged exposure to these solutions; (2) the membrane resistance is so low that small displacements of the feedback amplifier give large currents which may damage the fibre or the electrode; (3) it may take a long time to remove calcium from the neighbourhood of the membrane; and (4) the fibre is liable to fire continuously for many minutes.

From the results which have been described previously one might expect that a fibre in a calcium-free solution would be in a state in which the potassium conductance was fully turned on and the sodium-carrying system was largely inactivated. The records in Fig. 13 show that these predictions were only approximately fulfilled. Records 1*a* and 1*c* give the currents associated with pulses of ± 70 mV in 112 mM-Ca. Records 2*a* and 2*c* give the currents associated with the same pulse after 10 min in a Ca-free solution. It will be seen that, although the potassium conductance is larger in the resting condition, the fibre still rectifies. The existence of a phase of inward current after the end of the anodal pulse suggests that the sodium-carrying system can still operate provided inactivation is removed by previous hyperpolarization. On continued washing with Ca-free solutions, the fibre becomes more like an ohmic conductor. Records 3*a* and 3*c* were taken 20 min later and show less rectification and less inward current after the end of the anodal pulse. However, it will be seen that the maximum potassium conductance has decreased since the final level of outward current is smaller than previously. This suggests that there may be a loss of 'carrier' in low calcium, but the effect is not simply an irreversible decline because the fibre recovers much of its normal rectifying and regenerative properties on replacing 112 mM-Ca (records 4*a* and 4*c*). Similar results were obtained in other experiments though the change in rectification was sometimes more conspicuous (Fig. 14).

The tentative conclusions from these experiments are:

(1) that a squid fibre in zero calcium is in a refractory condition with a high potassium conductance and the sodium-carrying system largely inactivated;
(2) that changes in sodium and potassium conductance can still take place to a limited extent with only traces of calcium in the external medium;
(3) that fibres in 'zero' calcium slowly lose the 'carriers' which allow the membrane to undergo changes in sodium and potassium conductance, and
(4) that the 'carriers' seem to be replenished fairly rapidly when a high external [Ca] is restored.

In addition, it seems probable that the membrane loses much of its selective permeability and becomes a poor insulator in calcium-deficient solutions. This would account for the fall in resting potential which is often observed and for

the absence of polarization effects after the end of the cathodal pulse in zero calcium (see Frankenhaeuser & Hodgkin, 1956, p. 361).

Unpublished experiments with a conventional type of internal electrode indicate that although there may be little change in resting potential, squid fibres become inexcitable in zero calcium within 5–20 min (longer in *Sepia*). If the fibres are not kept too long in calcium-free solutions, the spike is restored within about 1 min by calcium concentrations greater than about 4 mM. The effect of calcium-free solutions on frog nerve fibres is described by Frankenhaeuser (1957). In this case, if adequate precautions are taken to remove traces of calcium, the fibres block in calcium-free solutions.

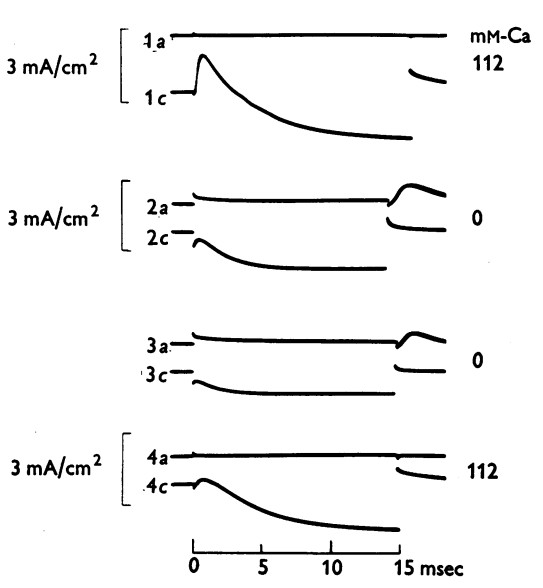

Fig. 13

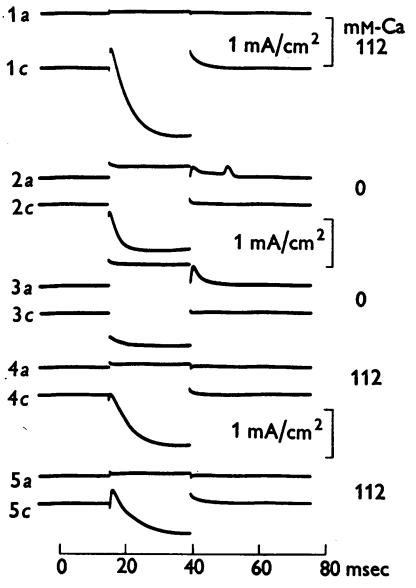

Fig. 14

Fig. 13. Membrane currents associated with rectangular pulses of amplitude 70 mV; hyperpolarizations labelled *a*, depolarizations labelled *c*; calcium concentrations as shown. The pulses were applied without any preceding hyperpolarization. Records 1*a* and 1*c* in 112 mM-Ca. Records 2*a* and 2*c* are taken 13 min after changing to a calcium-free solution. Records 3*a* and 3*c* after 33 min in calcium-free solution. Records 4*a* and 4*c* were taken 2 min after the solution was changed back to 112 mM-Ca. Axon diameter 714μ; temperature 5·2° C; solutions with $[Na]_0 + \frac{3}{2}[Ca]_0 = 560$ mM.

Fig. 14. Similar to Fig. 13 but with a different axon and with pulses of amplitude ± 56 mV. 1*a* and 1*c* in 112 mM-Ca; 2*a* and 2*c* taken 3 min after calcium-free solution was applied; 3*a* and 3*c* after 12 min in calcium-free solution; 4*a* and 4*c* were taken 8 min after returning to 112 mM-Ca; 5*a* and 5*c* after 11 min in 112 mM-Ca. The pulses start from an 11 mV anodal polarization in records 5*a* and 5*c*, from the resting potential in the other records. Axon diameter, 693μ; temperature, 6·5° C. The hump after the end of the pulse in 2*a* is a propagation artifact, see p. 219. This fibre had previously been used for other measurements.

Magnesium

Since there is evidence (e.g. Gordon & Welsh, 1948; del Castillo & Engbaek, 1954) that the effect of magnesium on threshold is similar to that of calcium, the effects of a solution containing 112 mM-Mg (and no Ca) were compared with the standard 112 mM-Ca solution. The axons were found to deteriorate rather rapidly in the Mg solution and it was not easy to make a quantitative comparison between Ca and Mg. Such evidence as we have indicates that (apart from any long-term effects of Ca-free Mg solutions) Mg has a stabilizing action like that of Ca but is somewhat less effective. Thus the potential at which the sodium conductance was one-tenth maximal was found to be 5 mV nearer the resting potential in the Mg solution. Taking the shift in the $g_{Na} - V$ curve as 15 mV between 112 and 22 mM-Ca (Table 3) it follows that the 112 mM-Mg solution is roughly equivalent to a solution containing 65 mM-Ca.

TABLE 8. Effect of solutions containing Mg on the time constant (τ_2) with which sodium conductance is 'shut off' under an anode (cf. Table 4)

			112 mM-Ca		112 mM-Mg	
Expt.	V (mV)	Pulse (mV)	Temp. (° C)	τ_2 (μsec)	Temp. (° C)	τ_2 (μsec)
(1)	+40	−128	6·1	80	6·9	154
	+40	−128	6·3	93	—	—
(2)	+41	−127	7·7	90	7·2	180
	+41	−127	7·0	90	—	—

Membrane potentials given relative to resting potential, depolarization negative.

In two experiments the rate at which the sodium conductance shuts off under an anode was compared in 112 mM-Ca and 112 mM-Mg. These results, which are given in Table 8, suggest that Mg is about half as effective as Ca.

Further evidence on the relative effectiveness of Ca and Mg will be given in a subsequent paper (Frankenhaeuser, Hodgkin & Keynes, in preparation). These experiments indicate that an artificial sea water containing 11 mM-Ca and 55 mM-Mg has about the same effect on the spike of the squid fibre as a solution containing 44 mM-Ca. The Ca:Mg ratio deduced from this result is 1:0·6; those obtained in the present work are 1:0·6, from the shift in the $g_{Na} - V$ curves and 1:0·5 from the change in rate constant.

DISCUSSION

Many of the results in this paper are summarized by saying that changes in calcium concentration and changes in membrane potential have similar effects on the systems which allow sodium and potassium to move through the membrane during the spike. This applies, qualitatively, to the variation of sodium and potassium conductance with membrane potential and to the rates of change of conductance at different membrane potentials. It also applies to the

variation of inactivation with membrane potential and to the rate of change of inactivation. The conclusion can be made quantitative by saying that a fivefold increase in calcium is equivalent to a hyperpolarization of 10–15 mV. However, in this case one must make the reservations that calcium appears to have a disproportionately large effect on the rate at which sodium conductance shuts off under an anode and that the shift in the inactivation curve may be less than the shift in the sodium conductance curve.

The action of calcium on the system controlling sodium and potassium permeability probably explains its effect on excitability. Thus a nerve becomes more excitable in low calcium, because a smaller depolarization is required to increase the sodium conductance to the critical level at which the inward sodium current exceeds the outward current carried by potassium and other ions. Some of the other effects of low calcium, for example the increased tendency to oscillate (Monnier & Coppée, 1939; Arvanitaki, 1939), the low membrane resistance (Cole, 1949) and the tendency to give anode-break responses, are explained partly by the shift in the sodium conductance curve and partly by similar changes in the potassium conductance and inactivation curves.

Two kinds of hypothesis have been advanced to account for the effects of calcium. One suggestion, made to us by Mr A. F. Huxley, is that calcium ions may be adsorbed at the outer edge of the membrane and thereby create an electric field inside the membrane which adds to that provided by the resting potential. In this way, adsorbed calcium ions might alter the distribution of other charged particles inside the membrane without changing the over-all potential difference between inside and outside. A variant of the idea is to say, that in low calcium these ions dissociate from negative charges and that the latter counteract the stabilizing effect of the field which is present normally. This idea accounts satisfactorily for the parallelism between changes in calcium concentration and changes in membrane potential and for most of the other effects described here. It does not explain the large effect of calcium on the rate at which sodium conductance is shut off under an anode, nor does it account for the increased calcium entry associated with the conduction of impulses (Flückiger & Keynes, 1955; Hodgkin & Keynes, 1957).

A second suggestion, based on the similarity between the action of calcium and changes of membrane potential, is that the increase in permeability underlying electrical activity occurs because depolarization removes calcium ions from sites or carriers in the membrane (see Hodgkin *et al.* 1949; Brink, 1954). The simplest form of this hypothesis is that Ca^{2+} is removed by the purely physical effect of the electric field on the distribution of Ca^{2+} in the membrane. Thus one might imagine that Na^{+} crossed the membrane through special holes which were blocked when occupied by Ca^{2+} but selectively permeable to Na^{+} when Ca^{2+} was first removed. If the sites occupied by Ca were

near the inner edge of the membrane and were accessible to external Ca but not to internal Ca, the proportion of time for which the holes would be open should be greatly increased either by reducing external Ca or by reducing the potential difference across the membrane. On this basis, the ratio of closed holes [CaX] to open holes [X] at equilibrium would be proportional to $[Ca]_0 \exp 2a\,EF/RT$, where E is the membrane potential difference and a is the fraction of it by which calcium ions are affected in moving from the external fluid to the sites where they act; a would be 0·5 if the calcium sites were half way through the membrane and nearly 1 if the sites were at the inside. Since $RT/F = 25$ mV and a must be less than 1, it follows that this hypothesis predicts an increase in membrane potential of at least 12·5 mV as being equivalent to an 'e-fold' increase in external calcium. For the theory to be at all plausible, the shift in membrane potential should probably not be less than 15 mV for an e-fold change. Our measurements indicate that 9 mV is equivalent to an e-fold change and are therefore incompatible with the theory. It might be thought that the difference was not important since the present measurements are not claimed to be exact. However, other evidence indicates that the estimate of 9 mV may be on the high side. Thus Weidmann's (1955) results with Purkinje fibres or Frankenhaeuser's (1957) with myelinated fibres indicate an equivalence of about 6 mV for an e-fold change; measurements of the threshold potential in *Sepia* axons give an average of 12 mV for an e-fold change at high calcium concentrations and 7 mV at low concentrations (Hodgkin & Keynes, unpublished). Further evidence against the simple theory of electrical removal of Ca is that, when $[Ca]_0$ is reduced, the rate of rise of sodium conductance increases more than would be expected on the basis of such a theory. The alternative to the simple theory is to suppose that calcium combines with carriers whose distribution depends on the membrane potential. This idea is clearly too speculative to be worth pursuing at present, but the general possibility that depolarization acts by removing Ca^{2+} from combination with a sodium carrier seems sufficiently plausible to keep in mind.

SUMMARY

1. Using the voltage-clamp technique, membrane currents were recorded in a squid giant axon surrounded by solutions containing different concentrations of calcium (or occasionally magnesium) together with the usual concentrations of Na, K and Cl. In order to start with the membrane in a fully resting condition, the cathodal pulses used to turn on the sodium or potassium permeability were superposed on a steady anodal polarization of 40–60 mV.

2. Decreasing the external calcium concentration, $[Ca]_0$, caused large and reversible increases in the inward sodium current associated with moderate depolarizations, but had relatively little effect on the currents associated with depolarizations of 60–120 mV.

3. When $[Ca]_0$ was reduced fivefold, the curve relating peak sodium conductance to membrane potential was shifted about 15 mV along the voltage axis, in a direction such that a smaller depolarization was required to increase the sodium conductance to a given size.

4. Reducing $[Ca]_0$ increased the rate at which the sodium conductance rose under a cathode and decreased the rate at which it declined under an anode.

5. In the steady state, reducing $[Ca]_0$ at constant membrane potential increased the proportion of the sodium-carrying system which was in an inactive or refractory condition.

6. Reducing $[Ca]_0$ caused large increases in potassium current at moderate depolarizations and shifted the curve relating potassium conductance to membrane potential along the voltage axis; a fivefold reduction of $[Ca]_0$ was roughly equivalent to a depolarization of 10–15 mV.

7. Reducing $[Ca]_0$ increased the rate of rise of potassium conductance under a cathode and shortened the delay with which the conductance rose.

8. Axons treated for some time with Ca-free solutions appeared to be in a refractory condition in which the potassium conductance was high and the sodium-carrying system largely inactivated.

9. Magnesium had similar stabilizing effects to calcium but was less effective.

10. Many of the actions of calcium can be summarized by saying that the effects of a fivefold reduction of calcium on the system controlling Na and K permeability are similar to those of a depolarization of 10–15 mV.

We wish to thank the Director and staff of the Laboratory of the Marine Biological Association at Plymouth for assistance and for the time spent in obtaining large specimens of *Loligo*. We also wish to thank Dr S. Weidmann, Mr A. F. Huxley and Dr R. D. Keynes for helpful discussion. The expenses of the work were met by grants from the Rockefeller and Nuffield Foundations and the Swedish Medical Research Council.

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